Environ. Sci. Technol. **2006**, *40,* 2678−2685

HPLC-DAD and Q-TOF MS Techniques Identify Cause of *Daphnia* Biomonitor Alarms in the River Meuse

CORINA J. DE HOOGH,*,† ARCO J. WAGENVOORT,‡,§ FRANK JONKER,‡ JAN A. VAN LEERDAM,† AND ARIADNE C. HOGENBOOM†

Department of Chemical Water Quality and Health, Kiwa Water Research, P.O. Box 1072, 3430 BB Nieuwegein, The Netherlands, and Evides Water Company Ltd., P.O. Box 59999, 3008 RA Rotterdam, The Netherlands

Several water companies in the Netherlands use a combination of specifically targeted compound analysis (HPLC-UV and GC-MS) and effect monitoring (continuous biotests) to monitor source water quality and to screen for unknown compounds. In spring 2004, the *Daphnia* biomonitor at Keizersveer monitoring station alongside the River Meuse recorded several alarms. In this study, the combination of HPLC-DAD and Q-TOF MS techniques was used to identify the so-far unknown microcontaminant related to this *Daphnia* alarm as 3-cyclohexyl-1,1-dimethylurea. The maximum concentration of this compound in the River Meuse at the time of the alarm was estimated to be 5 μg/L. The response of the waterfleas to this compound was confirmed with a short-term and a long-term verification test. The origin of the pollutant is still unknown. This paper shows that the combined application of on-line continuous biotests and advanced chemical analysis is an effective tool for the detection and identification of unknown, potentially hazardous compounds for surface water quality monitoring. Biological effect monitoring and specific compound analysis complement each other and together provide the best possible insight in rapid surface water quality changes.

Introduction

In the Netherlands, almost 40% of all drinking water is produced from surface water. Around 4 million people depend on the River Rhine as a direct or indirect source for their drinking water (*1*) and approximately 1.3 million people in the Rotterdam region are provided with drinking water originating from the River Meuse. Evides Water Company manages three artificial reservoirs in the Biesbosch wetland (Figure 1), which are fed with River Meuse water. The River Meuse is one of Western Europe's major rain-fed rivers, showing large fluctuations in discharge and quality. The reservoirs are therefore used for storage during times of low flow or high pollution levels in the river, and due to the long retention time of the water in the reservoirs (ca. 5 months), a significant improvement of water quality is achieved. Annually, 180 Mm^3 of reservoir water is used for drinking water production and industrial and agricultural purposes.

Due to large and rapid fluctuations in water quality, the water utilities using these surface waters need to monitor raw water quality closely in order to guarantee a continuous supply of high-quality drinking water. In case elevated levels of potentially hazardous contaminants are detected, they have to close the intake of source water or adjust the treatment process in time. For this, Evides operates an early warning system at Keizersveer monitoring station, located approximately 9 km upstream of its abstraction point (Figure 1). The early warning system consists of sensors for measuring temperature, pH, turbidity, electric conductivity, and dissolved oxygen levels, continuous biotests, and on-line chemical monitoring instruments. The chemical monitoring program screens for a number of known compounds of interest. For the on-line detection of unknown pollutants, e.g., herbicides, insecticides, and industrial chemicals, two on-line biomonitors, an algae-toximeter and a *Daphnia*-toximeter (both from bbe-Moldaenke, Kiel, Germany), have been installed.

The upstream location of this station allows for timely warning in case of a pollution event and continuous water quality monitoring at times when the intake of river water is stopped. The monitoring station is important in the selective abstraction policy and the multiple-barrier concept of Evides. Closing the intake of water has no immediate consequences for drinking water production, because the storage capacity ensures the availability of water to the production plants for several months.

The combination of effect monitoring techniques, such as continuous biotests, and specifically targeted compound analysis (HPLC-UV and GC-MS) has proven to provide the best possible water quality information for river water quality monitoring purposes (*2*, *3*). Different combinations of HPLC-UV, GC-MS (laboratory or field applications), and on-line continuous biotests using bacteria, algae, daphnids, mussels, or fish are most often used in the Netherlands. The application of on-line continuous biotests for water quality monitoring using *Daphnia* as a biosensor has been described in detail by Gunatilaka et al. (*4*).

In spring 2004, the bbe *Daphnia*-toximeter at Keizersveer monitoring station recorded several alarms. Although the cause of the alarms could not be identified, the intake of water from the River Meuse into the Biesbosch reservoirs was stopped for several days after each alarm. During the periods of the alarms, the results of on-line sensors at the monitoring station, as well as those of the on-line chemical monitors, were evaluated in order to identify a possible cause for the alarms. In addition, daily water samples from an on-site autosampler were analyzed using a combination of different analytical techniques.

For the detection of a wide range of polar water soluble compounds present in water, HPLC-MS-MS is a powerful technique (*5*, *6*). Although structural elucidation by HPLC-MS is possible and has been applied for many years (*7*, *8*), it is a difficult and time-consuming process due to low sensitivity in the full-scan mode and lack of libraries. Accurate mass measurements in combination with MS-MS can be used to solve these problems. With the use of a modern analytical technique, quadrupole time-of-flight mass spectrometry (Q-TOF MS), it is possible to determine more accurately the mass of unknown compounds. With the

* Corresponding author phone: +31 30 60 69 597; fax: +31 30 60 61 165; e-mail: corina.de.hoogh@kiwa.nl.
† Kiwa Water Research.
‡ Evides Water Company Ltd.
§ Currently affiliated with AqWa, Goes, NL.

10.1021/es052035a CCC: $33.50

Published on Web 03/08/2006

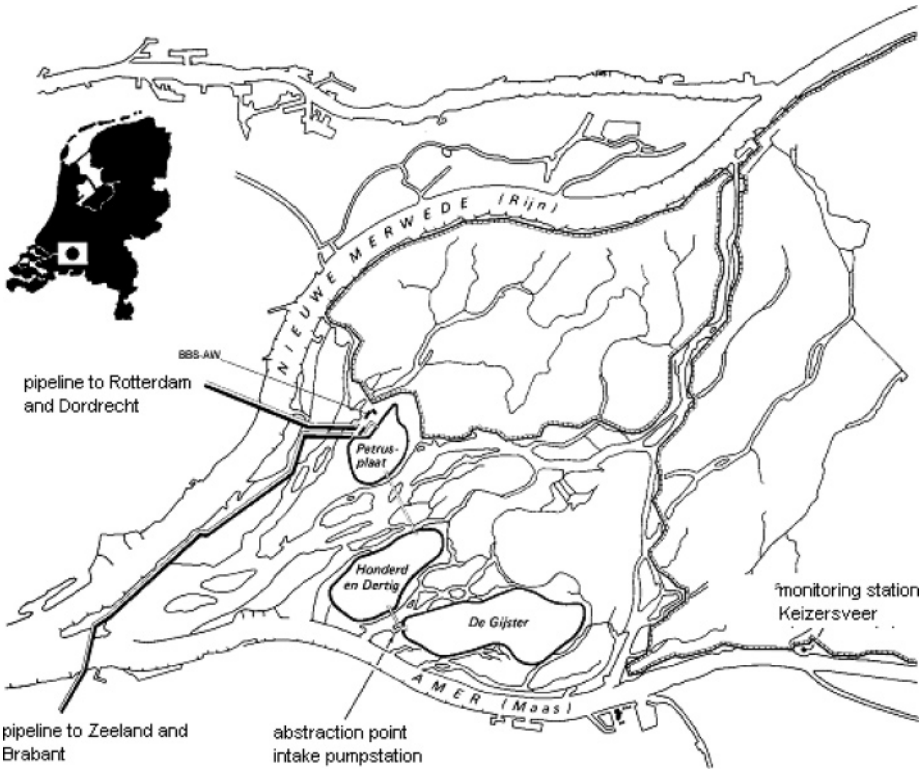


FIGURE 1. Location of the Biesbosch reservoirs, the intake of Evides, and the monitoring station Keizersveer.

accurate mass, the number of possible chemical formulas is drastically reduced.

Another relevant technique for the detection of a wide polarity range of compounds present in water is HPLC diode array detection (HPLC-DAD), which is often used for water analysis. With this technique, the spectra of all eluting (UV absorbing) organic compounds are acquired. In this study, the combination of both HPLC-DAD and HPLC-Q-TOF MS techniques was used for the detection and identification of an unknown micro-contaminant in water samples from the River Meuse that were collected during the *Daphnia* biomonitor alarms. This paper describes the alarm events, the characterization of this unknown substance, and verification tests with the *Daphnia* biomonitor.

Material and Methods

Chemicals. Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA). Gradient grade acetonitrile (Riedel-de Haën, Seelze, Germany) was used as an organic modifier. The eluent was deaerated with helium (99.999% pure, Hoekloos, The Netherlands) and placed under a constant pressure of 0.2 bar. Analytical grade ammonium acetate, fenuron, chloroxuron, and phosphoric acid were obtained from Mallinckrodt Baker (Deventer, The Netherlands). 3-Cyclohexyl-1,1-dimethylurea ($C_9H_{18}N_2O$, CAS-number 31468-1-29) was obtained from Sigma Aldrich (Zwijndrecht, The Netherlands).

Principle of the *Daphnia*-Toximeter. The bbe *Daphnia*-toximeter was developed to perform on-line toxicity tests using *Daphnia magna* as a test organism. The principle of measurement is based on two static biological test procedures: (*i*) ISO 6341, determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea)—Acute toxicity test (ISO 1996); and (*ii*) ISO 10706, water quality—determination of long-term toxicity of substances to *Daphnia magna* Straus (Cladocera, Crustacea) (ISO 2000).

Based on these procedures, the method allows for on-line registration of acute behavioral changes of the test organisms. The behavior of *Daphnia magna* is recorded by digital image processing and is continuously evaluated. Swimming behavior of *D. magna* in unpolluted water is marked by relatively calm movements with a more or less constant speed. Under influence of pollutants, the behavior changes to hypo- or hyperactivity, according to the type, concentration, and reaction time of the compounds. If the behavior of the daphnids deviates significantly from the observed behavior in a pre-defined previous period, an alarm is generated. In this way, the user will receive information about the deterioration of water quality long before any daphnids die. A detailed description of the operation principles of the *Daphnia* biomonitor, the behavioral parameters monitored, and the alarm detection algorithms is given in refs *9* and *10*.

Before exposing the daphnids to the sample water, the water was filtered and degassed. A 100 μm filter was used to prevent clogging of the biomonitor by larger particles, and at the same time keep the smaller particles available for uptake by the daphnids. *Daphnia* magna can take up particles to a maximum size of about 50 μm (*11*), and these particles can significantly influence the toxic response of the animals to certain chemicals (*12*). Successive heating of the filtered water to 25 °C and re-cooling to 20 °C generated degassing and more generally ensures a constant exposure temperature all year round. The measuring chamber is refreshed at least once every minute.

Alarm-detection, expressed as toxicity-index using the ToxIndex algorithm, is performed by using the pre-defined parameter-set for normal sensitivity of the instrument from bbe Moldaenke (Kiel, Germany). To these settings an adjustment was made for the mortality parameter. In case 50% or more daphnids should die, the system at Keizersveer will always record an alarm. To increase the uptime of the system and to perform additional data evaluation, a daily check is performed. During this check, evaluations are made by interpreting combinations of behavioral parameters such as swimming speed, swimming height, fractal dimension (indication of the swimming trajectory of the daphnids) and distance between, and comparing these parameters with the

reference behavior of *D. magna*. To allow for this type of data evaluation, the reference behavior of *D. magna* at Keizersveer was determined using the data of measurements of *D. magna* exposed to River Meuse water collected over a period of more than a year (*13*). Ten 2-day old *D. magna* are inserted in the system for a maximum of 4 days before being replaced. To generate reliable and reproducible results of the *Daphnia* biomonitor, a quality assurance system has been developed and implemented. This quality assurance system consists of standardized protocols for breeding daphnids, operation procedures, regular maintenance of the biomonitor, and data evaluation. The daphnids are examined macro- and microscopically on a regular basis. During the weekly service and after an alarm, a control sample (5 mg/L NaCl in 100 μm of filtered river water) is measured, to determine whether the total configuration is functioning properly.

Sample Collection and Preparation for Chemical Analysis. To determine chemical compounds related to the *Daphnia* alarms, two alarm samples and two unsuspected Meuse surface water samples were analyzed. To collect these composite grab samples, an autosampler at Keizersveer monitoring station adds 52 mL of sample water to a 5000 mL bottle every 15 min, thus collecting 5 L of sample water over 24 h.

For the first alarm period, River Meuse water samples from March 19 and 22, 2004 and regular Meuse water samples from March 17 and 25 were analyzed. For the second alarm period, River Meuse water samples from April 18, 19, 20, and 26, together with unsuspected Meuse water from June 15 were analyzed. All samples were filtered over 0.45 μm regenerated cellulose material RC 58 (Schleicher and Schuell). After filtration, fenuron (1.0 μg/L) and chloroxuron (1.0 μg/L) were added to the water samples as an internal standard. These internal standards are also used in the routine HPLC-UV screening method; one shows up at the beginning and one shows up toward the end of the chromatogram, thus covering the entire range of interest.

Chemical Analysis. The HPLC-DAD−Q-TOF-MS system consisted of a Gilson 232 autosampler, a Perkin-Elmer gradient HPLC-pump model 250, a Waters photodiode array detector model 996, and a Q-TOF micro mass spectrometer (Waters Micromass) equipped with a dual electrospray ionization (ESI) probe and LockSpray unit. The LockSpray ESI probe provided an independent source of the lock mass calibrant.

The preconcentration of the samples was carried out on a 20 × 3 mm i.d. column, packed with OASIS solid-phase material (Waters). OASIS is a porous copolymer [poly(divinylbenzene-*co*-*N*-vinylpyrrolidone)] with an adsorption capacity for both lipophilic and hydrophilic compounds [HLB, 25−35 mm, 73−89 Å pores, 800 m^2/g]. Solid-phase extraction (SPE) is one of the most popular techniques used in sample preparation prior to analysis by HPLC and GC, and is commonly used for environmental analysis (*14*). The preconcentration column was mounted on the injection valve of the autosampler and replaced the sample loop. The samples were preconcentrated online with an HPLC pump with a flow of 2 mL/min; the sample volume was 10 mL. A linear gradient of acetonitrile (10 to 100%) and water was used in 60 min with a flow of 0.7 mL/min. The analytical column was equilibrated after each analysis with a mixture of 13 mM ammonium acetate in 90% ultrapure water and 10% acetonitrile for 15 min.

The analytical column was a 250 × 4.6 mm i.d. Inertsil ODS-2, 5 μm materials from GL Sciences (Varian-Chrompack, Middelburg, The Netherlands). The guard column was 10 × 2 mm i.d. packed with pellicular C_{18} material, 25−35 μm (Varian-Chrompack). The analytical column and the guard column were maintained at a temperature of 21 °C in a column thermostat (W.O. Electronics, Applied Science Group, Emmen, The Netherlands).

The spectral resolution of the DAD was set to 1.2 nm, and the spectral range was from 200 to 350 nm. For the mass spectrometer, a lock mass was added by a LockSpray unit at a flow rate of 5 μL/min to allow for internal mass calibration and to provide optimal exact mass information (reference scan frequency: 20 s, cone voltage 30 V). For this purpose chloroxuron was used, which ionizes well in positive electrospray ionization mode (m/z 291.0900). The electrospray source conditions were as follows: capillary voltage 3 kV, cone voltage 30 V, source temperature 120 °C, desolvation gas temperature 250 °C, and desolvation gas flow 450 L/h. The MCP detector settings for full-scan acquisition were set to 2300 and 2500 V in the MS-MS mode (product ions). Pusher frequencies and cycle times were selected automatically. The resolution of the TOF was about 5500. Mass calibration was conducted with a 0.05% solution of phosphoric acid in acetonitrile/ultrapure water (50/50 v/v).

The LC column effluent was split via a postcolumn splitter from 700 μL/min to 125 μL/min which was introduced into the source of the mass spectrometer. To obtain maximum structural information, the samples were analyzed with both full-scan and product-ion scan mode. The scan range in the full-scan mode was set from 80 to 800 Da. Product-ion spectra were generated at a collision energy of consecutively 15 and 25 eV and acquired in the mass range of 40−400 Da. Argon was used as the collision gas and the gas cell pressure in the collision cell was 6×10^{-5} mbar. Acquisition and data processing were performed with MassLynx 4.0 SP2 software.

Data Processing. For the determination of accurate masses, experiments were performed in the continuum mode and converted to centroid data in order to implement lock mass correction and obtain accurately mass measured spectra (*15*). No TDC dead time correction was applied during acquisition. Exact masses of the ions of interest were determined based on the averaged spectra, no background subtraction or smooth was needed. To ensure correct mass calibration, the exact mass of the internal standard compounds fenuron and chloroxuron was checked.

Based on the accurate mass, the (possible) elemental composition of the peaks of interest was calculated using the elemental composition tool within the MassLynx software. Parameter settings were as follows: C 0−40, H 0−100, N 0−10, O 0−15, P 0−5, even electron ions for the precursor ions, and odd and even electron ions for the product ions. The appropriate numbers of the elements Cl, Br, and S were determined from the specific isotope pattern and added if required. The double bond equivalent (DBE) parameter was used as an indicator of the stability (degree of π-electron conjugation) of the calculated elemental composition and was set dependent to the existence of an UV signal. There was no significant UV signal, so the DBE parameter was set from −0.5 to +4.0.

In this study, the calculated elemental compositions with a maximum deviation of 10 mDa from the measured exact mass were considered. To obtain a chemical formula, the Merck index, the NIST library, a private database containing about 2500 water pollutants (pesticides and other contaminants), and Internet sites such as Chemfinder (www.chemfinder.com) and Sigma-Aldrich (www.Sigma-Aldrich.com) were searched. The structures found in the libraries were evaluated based on the fragmentation patterns observed in the acquired product-ion spectra.

Risk Assessment. Two models were used to assess the (acute) toxicity of 3-cyclohexyl-1,1-dimethylurea. With the DEREK model (available from Lhasa Ltd., Leeds, UK) the structure of a chemical and its observed properties are extrapolated to predict the behavior of this chemical in the

TABLE 1. Alarms of the *Daphnia* Biomonitor at Keizersveer Monitoring Station (Meuse) and Intake Management of the Biesbosch Reservoirs in Spring 2004

period	average swimming speed of *Daphnia magna*	mortality (%)[a]	intake of Meuse water into Biesbosch reservoirs	dates of samples analyzed with HPLC-DAD–Q-TOF-MS
March 11–15	normal	none		
March 15–18	normal	none		17–3
March 18–22	not enough daphnids for data-evaluation[a]	50	March 19: STOPPED automatically	19–3
March 22	not enough daphnids or data-evaluation	50		22–3
March 22–23	increased	40		
March 23–25	increased	10		25–3
March 25–29	increased	20		
March 29 – April 1	normal	10	March 29: RESTARTED	
April 9–13	normal	none		
April 13–15	not enough daphnids for data evaluation	40		
April 15–19	increased	40		18–4; 19–4
April 19–20	increased	40	April 20: STOPPED based on expert judgment	20–4
April 20–21	at the start increased, later not enough daphnids for data evaluation	50		
April 21–23	normal	10	April 22: RESTARTED	
April 24–26	slightly increased	none		26–4
April 27–29	normal	none		
April 29 – May 3	not enough daphnids for data evaluation	60	April 29: STOPPED automatically	
May 3–4	increased	40		
May 4–6	normal	none	May 4: RESTARTED	
	normal	none		15–6

[a] At 50% mortality, an alarm is generated automatically; at lower mortality rates, expert judgment is required to identify an alarm based on mortality. In the measuring chamber, 10 daphnids are present. In case some of the daphnids die, a statistically significant change in other behavioral paramters cannot be calculated automatically anymore. The behavioral change then has to be determined by expert judgment.

human body. The ECOSAR model, developed and distributed by U.S. EPA, predicts (acute) toxicity of a chemical to aquatic organisms.

Verification Tests. Two verification tests were performed to confirm the response of the daphnids to 3-cyclohexyl-1,1-dimethylurea. In a short-term test of approximately 5 h an acute effect of the substance on the daphnids was determined. A long-term test of several days was performed to better mimic the pollution events in the river as they had occurred.

Before the start of each verification experiment, the performance of the *Daphnia* biomonitor was evaluated using a control sample test. The verification test was only started when data evaluation of the preceding days showed no behavioral changes of the daphnids, which could indicate any changes in river water quality.

For the verification tests an experimental setup was designed in which 3-cyclohexyl-1,1-dimethylurea was continuously dosed in 100 μm of filtered river water before it entered the *Daphnia* biomonitor. In the short-term verification test of approximately 5 h, the 3-cyclohexyl-1,1-dimethylurea concentration was increased stepwise from 10 to 24, 50, and finally to a calculated concentration of 58 μg/L. The final concentration during the short-term test was confirmed by chemical analysis of the outlet water from the *Daphnia* monitor as 66 ± 9.9 μg/L. In the long-term verification test, which lasted 66 h, the 3-cyclohexyl-1,1-dimethylurea concentration was increased from 3.5 to 10 and finally to a concentration of 29 μg/L.

Results and Discussion

Alarm Events. Table 1 shows the dates of the alarms from the *Daphnia* biomonitor and the actions which were taken as a result of these alarms. Three alarm periods can be distinguished: March 19, April 20, and April 29. The alarms were induced by an increased average swimming speed and mortality of the daphnids. The intake of water from the river into the reservoirs was stopped as a result of these alarms. Only after several days, the behavior of the daphnids had returned to normal again and mortality was no longer observed, so the intake of river water could be resumed.

Data evaluation of the first alarm (March 19) showed that the alarm was caused by 50% mortality of the daphnids. Due to this high mortality, the swimming speed could not be evaluated properly. Continuous monitoring showed that in the following days mortality decreased and swimming speed gradually recovered to its normal range again, after having been increased. After the pollution wave had passed, the intake of water was resumed on March 29.

Twenty-four-hour composite grab samples of March 17, 19, 22, and 25 were analyzed, to compare both unsuspected and suspected river samples. Routine HPLC-UV analysis showed no significant differences in background concentrations of known compounds compared to the unsuspected samples. The sensors for pH, temperature, oxygen levels, turbidity, and electric conductivity showed no abnormal results at the time of the alarm.

During the daily control on April 20, 2004, based on observations from three successive series, the combination of increased average swimming speed of the daphnids and their mortality were identified as an alarm. As mortality did not reach 50%, an alarm was not generated automatically. In the following series (April 20 to 21) an alarm was generated, due to a mortality of 50%. On April 22, the behavior of the newly introduced organisms in the *Daphnia* biomonitor was normal again and mortality was only 10% (which is a possible result of the handling of the daphnids), so the intake of river

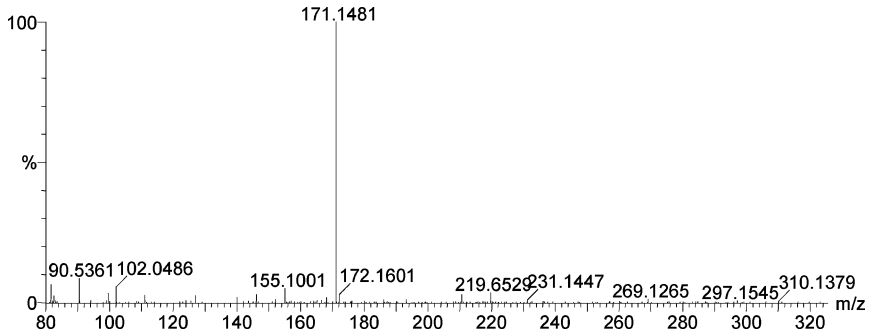


FIGURE 2. Full-scan mass spectrum of unknown peak in alarm samples of two alarm periods.

water was again resumed after having been closed since April 20.

The third alarm occurred on April 29, 2004, and was again identified by the combination of increased average swimming speed and mortality of the *Daphniae*. The intake had to be closed for 6 days until May 4.

Normally, in case of biomonitor alarms, chemical analysis of composite grab samples is performed using HPLC with UV detection. However, since the first results indicated that this method did not show much out of the ordinary, it was decided to analyze the available samples with HPLC-DAD−Q-TOF-MS, to find out whether this combination of methods could detect a possible cause for the alarms.

Identification. When plotting the maximum absorption of the HPLC-UV chromatograms in the wavelength range of 200−350 nm against the retention time, only small differences in the chromatograms of the alarm samples and unsuspected samples were observed. However, these differences did not allow for identification of single compounds. When comparing the HPLC−Q-TOF MS chromatograms of both the alarm samples and unsuspected samples using the base peak intensity (BPI) view, four significant peaks in the alarm samples were observed that were not, or only in low response, present in the unsuspected samples. The retention times of these peaks were 22.4 min (peak 1), 23.6 min (peak 2), 26.9 min (peak 3), and 32.1 min (peak 4).

Peak 1 is the most remarkable one, because it was observed in all water samples from the two investigated alarm periods but was completely absent in the unsuspected water samples. Moreover, the peak response in the suspected sample of March 22 was one of the highest in the LC-MS chromatogram obtained for this sample, with an estimated concentration related to the internal standard compound fenuron in the range of 1−5 μg/L.

Peaks 2, 3, and 4 were detected in water samples of both alarm periods but also detected in the unsuspected water samples (both March and June) at a low response. In earlier research performed by Kiwa Water Research (*16*, *17*), unknown peaks 3 and 4 were identified as hexa(methoxymethyl) melamine (HMMM) and isoproturon. HMMM is frequently used in the chemical industry as a cross-linker in thermotolerant coatings. Isoproturon is used as a herbicide. Both the UV and mass spectrum of peak 2 showed similarities to peak 3 and the compound corresponding to peak 2 was identified as penta(methoxymethyl)melamine ($C_{13}H_{26}N_6O_5$) (*18*). As only peak 1 could not be resolved using results from previous investigations, further research was focused on this specific peak. The Q-TOF full-scan mass spectrum of unknown peak 1 is shown in Figure 2.

The dominant ion in the full-scan mass spectrum with a mass of 171 Da corresponded most probably to a protonated molecule $(M + H)^+$, consequently, the molecular mass of the unknown compound is 170 Da. After internal mass calibration using the lock mass, the accurate mass of the protonated molecule was calculated as 171.1481 Da.

TABLE 2. Product Ions and Proposed Chemical Structures of the Unknown Compound Detected in *Daphnia* Alarm Samples of River Meuse Water near Keizersveer

product ion	elemental composition	theoretical mass	mass difference (mDa)	proposed chemical structure
89,077	$C_3H_9N_2O$	89,072	5	$NH_3-(CH3)_2-N-C=O$
72,050	C_3H_6NO	72,045	5	$(CH3)_2-N=C=O$
	$C_4H_{10}N$	72,081	31	
83,093	C_6H_{11}	83,086	6	cyclohexyl
55,061	C_4H_7	55,055	6	butyl

Based on the maximum mass tolerance of 10 mDa, a DBE range of −0.5 to +4.0 (no significant UV response) and an organic compound consisting of the elements C, H, N, O, and P (the elements Cl, Br, and S were excluded due to the lack of characteristic isotope patterns for these elements), two elemental compositions for the protonated molecule remained possible: $C_9H_{19}N_2O$ (accurate mass 171.1497, mass deviation −1.6 mDa, DBE = 1.5) and $C_{10}H_{19}O_2$ (accurate mass 171.1385, mass deviation 9.6 mDa, DBE = 1.5). To obtain additional information about the chemical structure, the product ions of precursor mass 171 were generated at collision energies of consecutively 15 and 25 eV.

Based on the accurate mass of the generated product ions, elemental compositions for each product ion were suggested as shown in Table 2.

After combining the results from both the full-scan and production mass spectra and extensive research, it was possible to extract one elemental composition ($C_9H_{18}N_2O$) for the unknown peak that fitted best with the obtained information.

To propose a chemical structure, chemical libraries such as Merck, NIST, Sigma-Aldrich, and several Internet sites were screened. In this screening, it was taken into account that the unknown compound showed no UV-absorption, most probably because of lack of aromaticity of the molecule. After comparing about 20 chemical structures of the correct chemical composition, it was concluded that two chemical structures (3-cyclohexyl-1,1-dimethylurea and 1-cyclohexyl-3-ethylurea) fitted with the mass spectral and UV information. To confirm the exact identity of the compound in the water samples, pure reference standards of these two chemicals were purchased and analyzed under analytical conditions identical to those of peak 1. This showed an identical LC retention time and MS product ions for both 3-cyclohexyl-1,1-dimethylurea and the unknown compound corresponding to peak 1, thus elucidating the chemical structure of this component.

In Figure 3, the chemical structures of the four identified compounds detected in River Meuse water during the *Daphnia* alarms are presented.

Amount and Origin of the Compound and Risk Assessment. After identifying the compound as 3-cyclohexyl-1,1-dimethylurea, its concentration in the source water was

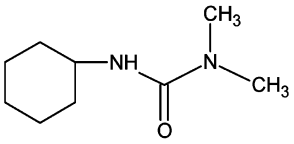
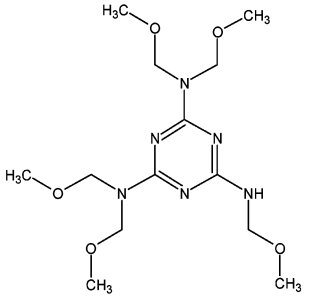
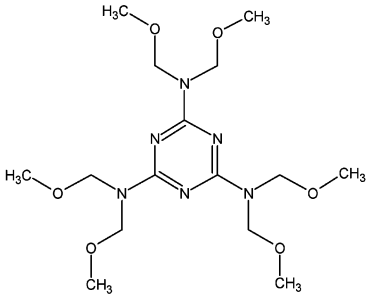
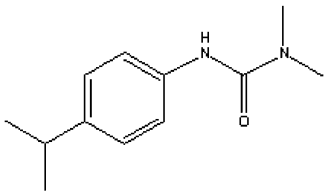
Compound number	Chemical name	Chemical structure	CAS number
1	3-cyclohexyl-1,1-dimethylurea		31468-1-29
2	Penta(methoxymethyl) melamine (PMMM)		?
3	Hexa(methoxymethyl) melamine (HMMM)		3089-11-0
4	isoproturon		34123-59-6

FIGURE 3. Chemical structures of four identified compounds in water samples of River Meuse water at the moment of a *Daphnia* biomonitor alarm.

calculated by using the internal standard fenuron and the pure reference compound. The maximum detected concentration during the alarm periods was calculated to be 5 μg/L. Considering that the flow of the River Meuse in the respective period was about 1000 m^3/s and the composite sample was taken over a period of 24 h, the total amount dosed or spilled was estimated to be around 450 kg. This amount of an organic substance in the water will not cause a water quality change that can be detected by sensors for pH, electric conductivity, oxygen, or turbidity, which is in line with the observations during the alarms.

3-Cyclohexyl-1,1-dimethylurea was patented most recently in the 1980s by a German company named Fahlberg, as one out of two compounds in a selective herbicide combination (*19*). However, no registrations for application of this herbicide or any production locations could be found in the entire European Union. In 1995, another patent naming this compound was filed by Mitsubishi Gas Chemical Co., Japan, regarding traction drive oils containing amides or thioamides for large traction coefficients (*20*). This patent document mentions the use of 3-cyclohexyl-1,1-dimethylurea in hydraulic fluids and lubricating oils. The fact that the presence of 3-cyclohexyl-1,1-dimethylurea was accompanied by an increased concentration of HMMM, which is used as a cross-linker in thermotolerant coatings, suggests that the presence of 3-cyclohexyl-1,1-dimethylurea could originate from a drainage of a hydraulic fluid. The exact origin, however, remains unclear.

With the help of toxicological models an attempt was made to find out whether 3-cyclohexyl-1,1-dimethylurea is acutely toxic to humans and aquatic organisms in general. The DEREK model indicates that 3-cyclohexyl-1,1-dimethylurea has a possible teratogenic effect in humans. The ECOSAR model predicts an LC_{50} of 180 mg/L for narcotic effects on *Daphnia* in a static test. However, the observed behavioral change in swimming speed of the daphnids during the alarms suggests a more specific effect of the compound rather than a narcotic effect. This, and the fact that the toxicological endpoint of this static test (mortality) is significantly different from that of the continuous test (behavioral change), could explain why the effects during the alarms were observed at a much lower concentration than the model predicts.

Another possible explanation for the difference between the theoretical toxicity as calculated by the models and the empirical toxic values determined by the alarm evaluation and the verification tests is the presence of particles in the sample water. Pollutants can be dissolved in the water or be attached to particles, thus forming two different exposure routes for the daphnids. This can significantly influence the toxicity of a compound (*12*).

Verification Tests. In Figure 4, the results of the short-term verification test are shown. As a result of the addition of 3-cyclohexyl-1,1-dimethylurea to river water, an increased swimming speed of the daphnids up to 0.63 cm/s was observed. This is well above the empirically determined reference level for swimming speed of 0.35−0.49 cm/s for

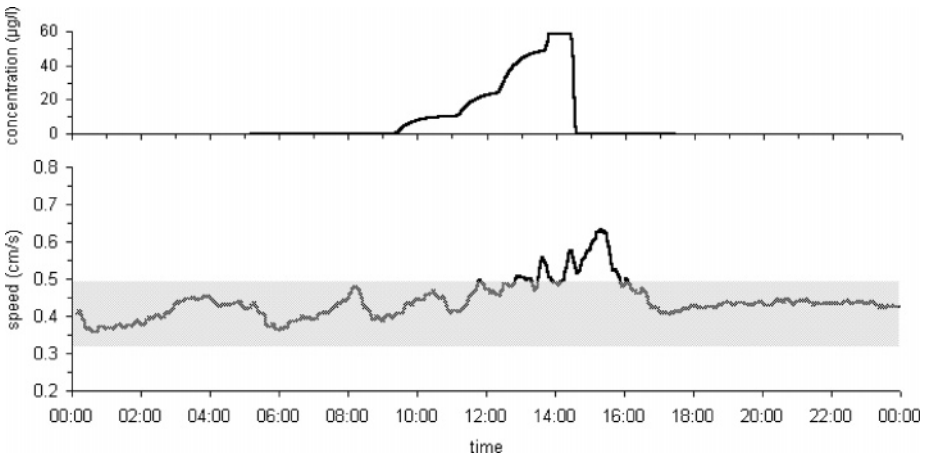


FIGURE 4. Swimming speed of *Daphnia magna* in the *Daphnia* biomonitor (bottom) during the short-term verification test with 3-cyclohexyl-1,1-dimethylurea (top) added to river water. Reference behavior of *Daphnia* is indicated by the gray bar.

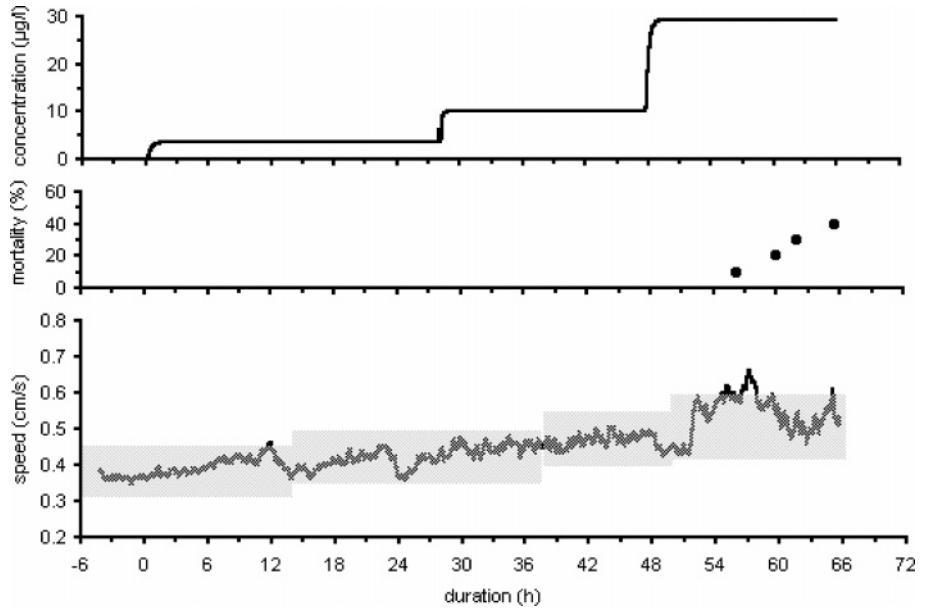


FIGURE 5. Swimming speed (bottom) and mortality (middle) of *Daphnia magna* in the *Daphnia* biomonitor during the long-term verification test with 3-cyclohexyl-1,1-dimethylurea (top) added to River Meuse water. Reference behavior of *Daphnia* changes over time due to growth, as is indicated by the gray bars.

Daphnia of the size used in the experiment. At the time the swimming speed was outside the reference values the concentration of 3-cyclohexyl-1,1-dimethylurea was approximately 30 μg/L. After the addition of 3-cyclohexyl-1,1-dimethylurea was stopped, the swimming speed remained at an elevated level for approximately $1^1/_2$ hours, then decreased again to the level of reference behavior. These results show that the response of *D. magna* to exposure to 3-cyclohexyl-1,1-dimethylurea in the normal matrix was delayed by approximately 1−2 h. This could indicate that an initial response of the daphnids could be expected at a level of 10 μg/L.

During the long-term test, the addition of 3.5 and 10 μg/L 3-cyclohexyl-1,1-dimethylurea during 1 day did not result in a direct response of the daphnids. Both mortality and increased swimming speed were observed in the long-term verification test at a concentration of 29 μg/L (Figure 5).

Both experiments illustrate that 3-cyclohexyl-1,1-dimethylurea added to normal river water can cause an increased swimming speed of daphnids, and the long-term test also shows that this compound can cause mortality. These responses were found at concentrations which are 3- to 10-fold higher than the concentrations found in the collected samples. It is possible that the concentration in these samples was lower due to dilution by the composite sampling regime. Moreover, higher concentrations may have occurred during days on which samples were not analyzed. Since the verification tests were performed with 100 μm of filtered water, the edible fraction of particles for daphnids was present during the tests, so the influence of particles in the toxicity can be excluded here.

During the first alarm period, 3-cyclohexyl-1,1-dimethylurea was found in combination with elevated levels of HMMM and PMMM. In the second alarm period, 3-cyclohexyl-1,1-dimethylurea was again found, this time in combination with isoproturon. This suggests that 3-cyclohexyl-1,1-dimethylurea is at least partly responsible for causing the *Daphnia* alarms, but the response could have been enhanced by additional pollutants. Considering that the alarms occurred three times in a short period of time (have not occurred anymore since) and the verification test showed a response at a 3- to 10-fold higher concentration, it is assumed that a specific spill during a short period of time is responsible for the alarms rather than a diffuse source of the pollution.

Acknowledgments

This project was sponsored in part by the joint research program BTO of the Dutch water utilities. Karina Pikaar-Schoonen (Evides) is acknowledged for her critical remarks on the data-evaluation of the biological monitors, Meral Brons-Westerink (Evides) and Ad Kuijpers (Aqualab) are acknowledgedfor their fast and accurate reaction and maintenance of the *Daphnia* biomonitor, and Minne Heringa (Kiwa Water Research) is thanked for bringing in her expertise on toxicological modeling. We acknowledge Ben Baardolf and Henk Ketelaars from Evides and Joep van den Boeke (Kiwa Water Research) for reviewing this article.

Literature Cited

(1) RIWA. *Year Report 2003*; ISBN 90-6683-109-X; Werkendam, 2003.
(2) Gunatilaka, A.; Diehl, P. A brief review of chemical and biological continuous monitoring of rivers in Europe and Asia. In *Biomonitors and Biomarkers as Indicators of Environmental Change 2*; Butterworth, F. M., Gunatilaka, A., Gonsebatt, M. E., Eds.; Kluwer Academic/Plenum Publishers: New York, 2000.
(3) Butterworth, F. M.; Gunatilaka, A.; Diehl, P. The Rhine/Danube monitoring paradigm: broader applications in sensor technologies. In *Proceedings of the International Symposium on Automation in Water Quality Monitoring—AutMoNet 2002*; Vienna, Austria, May 21−22, 2002; pp 299−305.
(4) Gunatilaka, A.; Diehl, P.; Puzicha, H. The evaluation of "Dynamic *Daphnia* Test" after a decade of use: Benefits and constraints. In *Biomonitors and Biomarkers as Indicators of Environmental Change 2*; Butterworth, F. M., Gunatilaka, A., Gonsebatt, M. E., Eds.; Kluwer Academic/Plenum Publishers: New York, 2000.
(5) Barcelo, D., Ed. *Applications of LC-MS in Environmental Chemistry*; Journal of Chromatography Library No. 59; Elsevier: Amsterdam, 1996.
(6) Niessen, W. M. A. *Liquid Chromatography−Mass Spectrometry*, 2nd ed.; Marcel Dekker: New York, 1999.
(7) Niessen, W. M. A. Structure elucidation by LC-MS. *Analusis* **2000**, *28* (10), 885−887.
(8) Marquet, P.; Venisse, N.; Lacassie, É.; Lachâtre, G. In-source CID mass spectral libraries for the "general unknown" screening of drugs and toxicants. *Analusis* **2000**, *28* (10), 925−934.
(9) Lechelt, M.; Blohm, W.; Kirschneit, B.; Pfeiffer, M.; Gresens, E.; Liley, J.; Holz, R.; Lüring, C.; Moldaenke, C. Monitoring of surface water by ultrasensitive *Daphnia* Toximeter. *Environ. Toxicol.* **2000**, *15*, 390−400.
(10) Schwörer, R.; Marten, M. Mathematische Sprungerkennung (Hinkley-Detektor und Steigungsoperatoren) im Einsatz bei der Erkennung von Messwert-Niveausprüngen bei kontinuierlichen Biotestgeräten; Deutsche Gesellschaft für Limnologie e. V. Tagungsbericht 2000; Kaltenmeier Söhne: Krefeld, 2000.
(11) Burns, C. W. The relationship between body size of filterfeeding Cladocera and the maximum size of particle ingested. *Limnol. Oceanogr.* **1968**, 675−678.
(12) Stratton, G. W.; Corke, C. T. Interaction of permethrin with *Daphnia magna* in the presence and absence of particulate material. *Environ. Pollut. (Series A)* **1981**, *24*, 135−144.
(13) Wagenvoort, A.; Pikaar-Schoonen, K.; De Hoogh-Carpentier, C. J.; Ketelaars, H. A. M. Algae and waterfleas are guarding the water quality of water used for drinking water in the south-west of The Netherlands. H_2O **2003**, *22*, 21−24 (in Dutch).
(14) Hennion, M.-C. Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography. *J. Chromatogr., A* **1999**, *856*, 3−54.
(15) Hogenboom, A. C.; Niessen, W. M. A.; Little, D.; Brinkman, U. A. Th. Accurate mass determinations for the confirmation and identification of organic microcontaminants in surface water using on-line solid-phase extraction liquid chromatography electrospray orthogonal-acceleration time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1999**, *13* (2), 125−133.
(16) Bobeldijk, I.; Stoks, P. G. M.; Vissers, J. P. C.; Emke, E.; van Leerdam, J. A.; Muilwijk, B.; Berbee, R.; Noij, Th. H. M. Surface and wastewater quality monitoring: combination of liquid chromatography with (geno)toxicity detection, diode array detection and tandem mass spectrometry for identification of pollutants. *J. Chromatogr., A* **2002**, *970*, 167−181.
(17) Bobeldijk, I.; Vissers, J. P. C.; Kearney, G.; Major, H.; van Leerdam, J. A. Screening and identification of unknown contaminants in water with liquid chromatography and quadrupole-orthogonal accelaration-time-of-flight tandem mass spectrometry. *J. Chromatogr., A* **2001**, *929*, 63−74.
(18) Nielen, M. W. F.; van de Ven, H. J. F. M. Characterization of (Methoxymethyl)melamine Resin by Combined Chromatographic/Mass Spectrometric Techniques. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 74−81.
(19) Fahlberg-List. Selective herbicide combinations. Patent no. DD 218265, 1985.
(20) Mitsubishi Gas Chemical Co. Traction drive oils containing amides or thioamides for large traction coefficients. Patent no. JP 07258669, 1995.

Received for review October 14, 2005. Revised manuscript received January 23, 2006. Accepted February 1, 2006.

ES052035A